

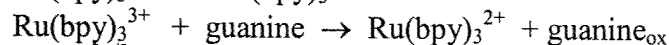
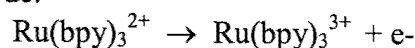
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13. ABSTRACT (Maximum 200 words) Report developed under SBIR contract for topic A01-032. We have demonstrated the feasibility of the use of mediated electrochemical detection of unmodified nucleic acids in a flow-through format. This detection method is rapid and sensitive, and easily implemented in a portable unit because of simple instrumentation required for the measurement and low power requirements. In addition, direct detection of unmodified nucleic acids avoids the use of complicated sample purification procedures, the introduction of exogenous labels and the use of labile reagents. In this proof-of-principle study, target nucleic acid was captured on beads or in a microfluidic channel and detected by oxidation of guanine residues after being heat released from the capture probe. Model target used in this study, ApoA1 mRNA from hepG2 cells (976-mer, 336 guanines) was detectable at 1 fmol input concentration in the present configuration. In addition to military applications, development of this unit would provide a system useful for prompt detection of bio-terrorist attacks. The unit would also find wide application for the detection of biological targets in medical diagnostics, point-of-care clinical settings, food production, environmental monitoring, agriculture, and various industrial settings.					
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This project was a proof-of-principle effort to examine the feasibility of adapting Xanthon's mediated electrochemical detection of nucleic acids to a format suitable for use as a hand-held biosensor. Nucleic acid detection system used in this study is based on the catalytic oxidation of guanine residues by a tris(bipyridyl) ruthenium (II) ($\text{Ru}(\text{bpy})_3^{2+}$) mediator^{1,2,3}. This detection method does not require the attachment of a label to the nucleic acid target and has been shown to have high sensitivity due to the catalytic nature of the oxidation reaction. $\text{Ru}(\text{bpy})_3^{2+}$ mediator transfers electrons from guanine to a tin-doped indium oxide (ITO) working electrode:



The $\text{Ru}(\text{bpy})_3^{2+/3+}$ couple is an excellent mediator for use in this system because its standard reduction potential (1.1V vs. Ag/AgCl) is almost identical to that of guanine. Similarly, ITO is the preferred electrode material as it is able to support the high positive potential necessary for guanine oxidation.

The work on development of a portable DNA biosensor unit was divided into two primary areas: development of the Hybridization Module and development of the Detection Module.

1. Hybridization module

Three approaches for implementation of the hybridization module have been examined:

- Active hybridization
- Hybridization using probe-coated magnetic beads
- Hybridization in microfluidic channels.

Each of these approaches was evaluated in terms of its ability to deliver a hybridization reaction that is highly efficient, rapid, specific and allows for release of the hybridized target from the immobilized probe to allow it to be detected by the Detection Module.

1.1 Active hybridization

Since the device to be designed in this work needs to operate in the field and allow for rapid detection of pathogens present in small quantities in the sample, the hybridization event has to be fast and highly efficient. The use of active, or electronically driven hybridization was explored as it has been shown to meet these requirements^{4,5}. In this approach, nucleic acid probe molecules were immobilized at platinum (Pt) electrodes and a high positive potential applied to attract the negatively charged nucleic acids in the sample to the immobilized probe. This attraction potential was followed by application of a negative potential to the electrode to remove non-hybridized material at the surface.

Two approaches to implementing active hybridization were attempted. In the first approach, platinum electrodes were fabricated electrodes and DNA probes deposited directly onto the electrode surface. Probes were coupled to isocyanato silane using standard Xanthon probe coupling chemistry to provide covalent attachment of the probes to the electrode. This probe attachment method lacked sufficient probe stability for use under the conditions of the assay.

The second approach used an acrylamide gel for probe coupling and immobilization. Attempts were made to couple acrylamide-modified oligonucleotide probe to the gel matrix in either TBE or TE buffers. The probe was labeled with ³²P to permit the amount of probe immobilized in the gel to be quantitated. Following preparation,

the gels were conditioned by immersion in 50 mM histidine buffer for 1 hour. During incubation approximately 30% of the probe was lost from the gel regardless of the buffer used. An additional 20% of the probe was removed from the gel upon application of a -1.5 V bias to the gel for 10 minutes. labeled probe was used to quantify the amount of probe immobilized in the gel. After the gels were prepared, they were conditioned by immersion in 50mM histidine buffer for 1 hour. Approximately 30% of the probe was lost from the gel regardless of the buffer used and an additional 20% of the probe was removed from the gel upon application of a -1.5V bias to the gel for 10 min. Hybridization of target to the probe remaining immobilized in the gel was attempted using electrochemical control to drive the 1.0 μ M 32 P-labeled 51-mer oligonucleotide in either 50mM histidine buffer or 500mM NaCl into the gel. Following application of the positive potential target solutions were removed and the electrochemical cell rinsed. A negative potential was then applied to the gel for 5 minutes to remove non-hybridized target. While oligonucleotide target can be drawn into and removed from the gel, complementary target could not be discriminated from non-complementary target indicating a lack of specific hybridization between the immobilized probe and the target nucleic acid.

In conclusion, our limited efforts to develop an active hybridization capture electrode array were unsuccessful. The possibility of successfully executing an active hybridization strategy cannot be eliminated based on this work as such strategies have been successfully implemented by others. Continued effort was, however, beyond the financial and time constraints of this project and alternate configurations were examined so that the project could move forward.

1.2 Hybridization using probe-coated magnetic beads

The use of magnetic beads for immobilization of nucleic acid probes for target hybridization is well established⁶. The main advantages of this method are the solution-like hybridization kinetics and the ease of manipulating the beads with magnets. Dynal beads with a polydT sequence were used to capture and immobilize an ApoA1 probe having a polydA linker. The probe-derivatized beads were then used to capture ApoA1 mRNA target (974 nucleotides and 336 guanines) or a shorter synthetic 51-mer DNA target out of solution. The short target was radiolabeled to monitor the hybridization efficiency on the bead as well as the release of the hybridized target using heat. Figure 1 shows representative data obtained using beads for capture and release of the 51-mer targets. In these studies, targets were used that were either complementary to the 18-mer probe immobilized on the beads, or were a complete mismatch for the probe. In Figure 1 the data for the complementary target (blue bars) show that approximately 50% of the target is removed from the solution during incubation with probe-coated beads and that approximately 80% of the hybridized target is released from the beads following application of heat. Little or no mismatch target (red bars) was removed from solution and levels of label are at background following release by heat. This graph illustrates that hybridization using magnetic beads is an efficient and specific process. The hybridization experiments were completed in 30 minutes.

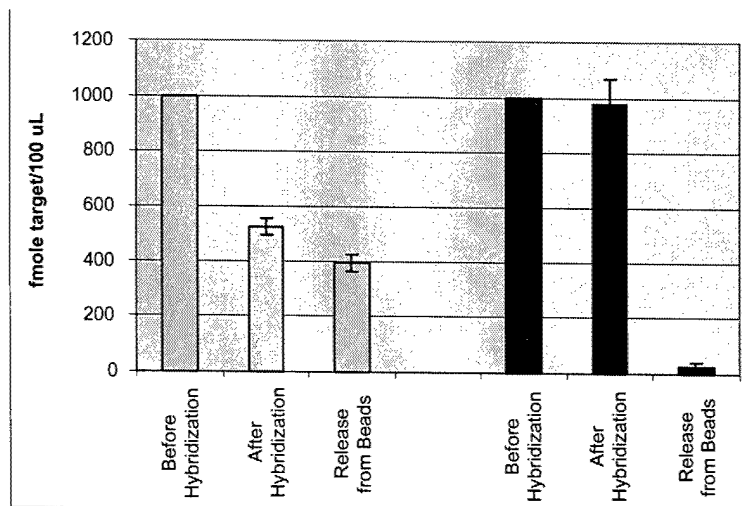


Figure 1. Efficiency and specificity of hybridization using Dynal magnetic beads.

This approach was also shown to be very efficient at capturing the ApoA1 mRNA target (974-mer). In this system the capture and release of ApoA1 target were monitored using mediated electrochemical detection in a flow-through cell as described below. Figure 2 shows representative data from a capture and release experiment with electrochemical detection. In this experiment, ApoA1 mRNA at an input concentration of 400 fmol/20 μ L solution was mixed in with the beads having immobilized complementary probe and incubated at room temperature for 30 minutes. Following hybridization, the beads were held with a magnet and washed three times. The final wash solution was injected in the flow-through cell as a control (first three peaks in the figure). The small signal observed is due to the non-bound mRNA being removed by the wash and can be eliminated by a more thorough wash sequence. The second set of three peaks was obtained after the beads have been heated to 100 $^{\circ}$ C for 5 minutes to release the captured mRNA into the supernatant solution. An aliquot (20 μ L) of the solution was removed and injected into the flow-through cell. The signal obtained corresponds to approximately 50 fmol when compared to the calibration curve data on the flow-through cell, indicating that not all of the mRNA is recovered in this process. This could be a result of an insufficient amount of the probe molecules that are required to capture the target and, if so, this can be overcome by increasing the number of beads and/or probe molecules on the beads to ensure full capture and recovery of the mRNA. Alternate bead chemistries can be used - for example, beads derivatized with streptavidin are known to capture biotinylated probe with high efficiency.

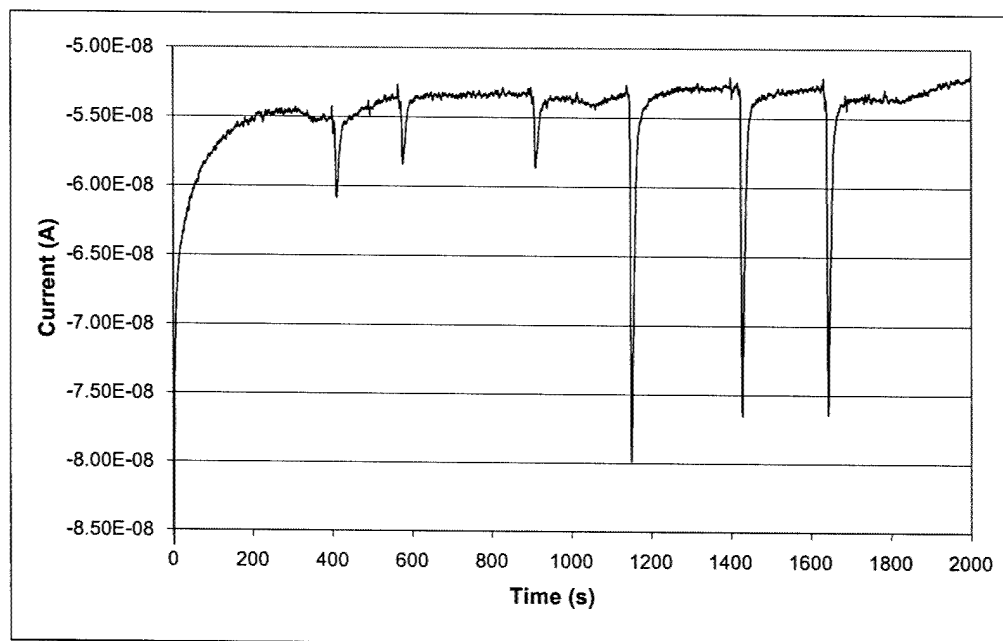


Figure 2. Electrochemical detection of the ApoA1 target in the flow-through system following its release from Dynal beads.

1.3 Hybridization in microfluidic channels

The speed and efficiency of hybridization in microfluidic channels has been demonstrated by numerous investigators^{7,8,9}. In this configuration probe can be immobilized on the walls of the channels or on a substrate that fills the channels. In either case, the small solution volume and ready availability of a high density of probe molecules create very favorable conditions for hybridization. The option of using microfluidic channels etched in glass for the present system was explored. The channels were obtained from Micralyne (Alberta, Canada) and coated with the standard Xanthone isocyanato silane - amine terminated probe. The coupled probe was

covalently attached to the glass substrate and excess material flushed out of the channel. Radiolabeled 51-mer synthetic DNA target was injected into the channel and the radioactivity followed as described above to assess the efficiency of target hybridization and release in this system. This hybridization format was found to allow for specific hybridization. However, due to the time constraints, hybridization and release efficiencies were not determined in this study.

2. Detection of nucleic acids in a flow-through format

The main advantage of detecting nucleic acids using a flow-through cell over stationary solution electrochemistry is that the solution is flowed across the electrode surface so that the analyte is brought to the electrode by convection instead of diffusion. Convection is a much more efficient way of delivering the analyte to the electrode surface and should allow for a significant enhancement of signal in comparison with solution electrochemistry¹⁰.

A flow-through system with a thin layer flow-through ITO cell has been assembled using commercially available components. The working electrode is held at a constant potential that is sufficiently high to oxidize Rubpy and guanine. The electrode area is 35 mm² and the cell volume is ca. 15 μ L. A wide variety of parameters were explored for optimal sensitivity and reliable performance. These variables include potential applied, flow rate and Rubpy concentration. The area of the working electrode was also varied from 3.14×10^{-4} cm² to 3 mm².

The preliminary experiments were performed using dGTP and dATP as a control. Figure 3 shows representative data obtained using the flow-through setup. As expected, the signal increases as the amount of guanine in the sample is increased. The control samples containing adenine do not give any extra signal above background, in agreement with numerous studies performed both both at Xanthon and H.H. Thorp's lab at UNC-Chapel Hill.

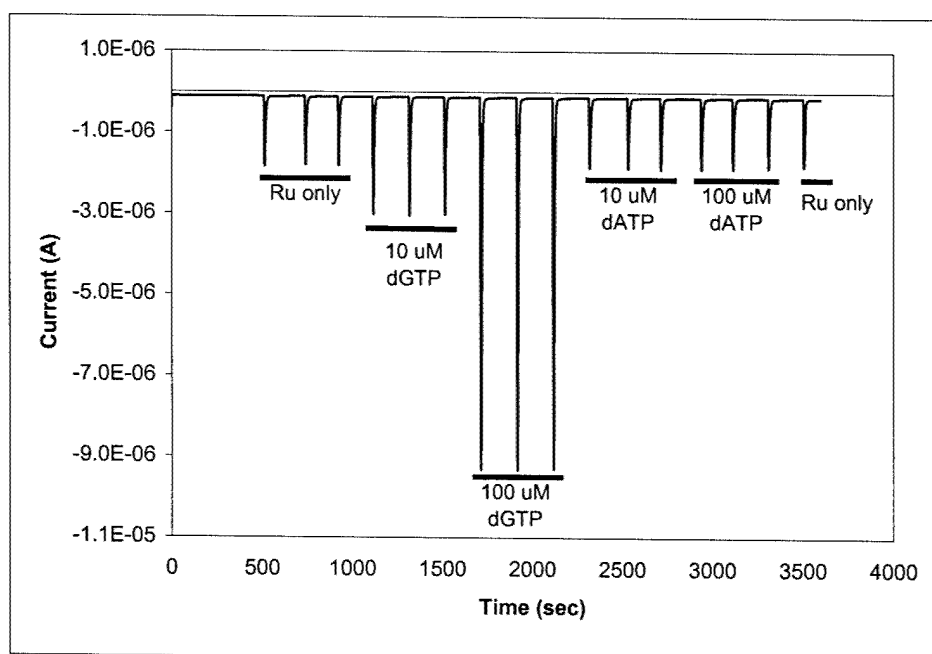


Figure 3. Detection of guanine in the flow-through system.

The next phase of the project involved detection of the mRNA targets using the flow-through setup. Initially, Rubpy was co-injected with the ApoA1 target. A representative data set is shown in Figure 4.

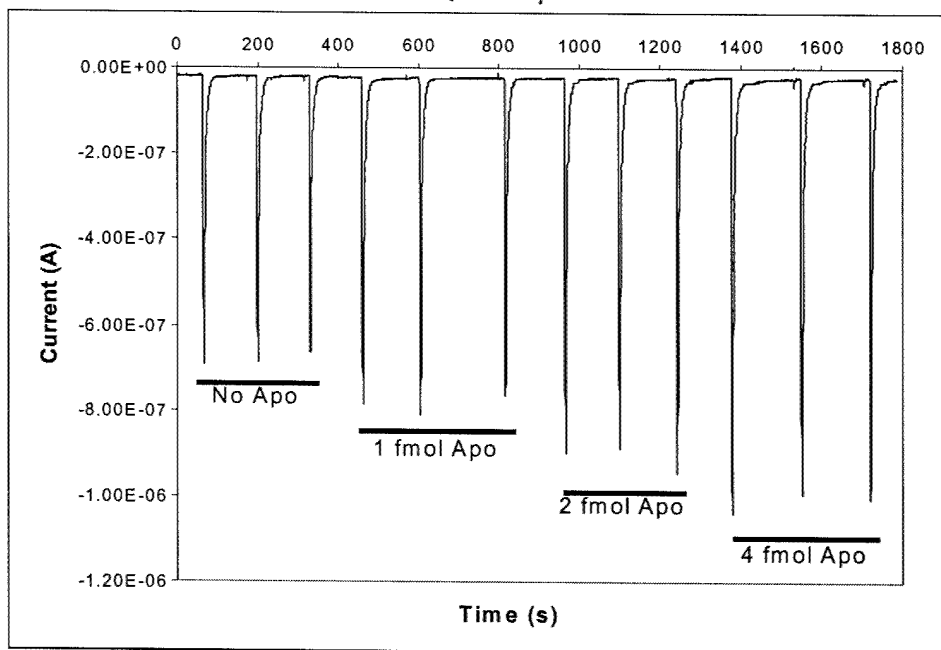


Figure 4. Detection of the ApoA1 target in the flow-through system with co-injected Rubpy.

Some of the parameters examined during development to optimize performance of the system for detection of mRNA target are listed below:

Influence of the potential applied:

Using large electrodes, potentials of 1250mV or less did not allow discrimination of 4 fmol ApoA1 mRNA over the 10 μ M Ru(bpy) background. Potentials of 1350 and 1400mV gave large background currents and large charging currents. 1300mV allowed discrimination of mRNA down to 1 fmol.

Influence of Ru(bpy) concentration:

Ru(bpy) concentrations of 1, 5, 10 and 20 μ M were used on large electrodes. At a Ru(bpy) concentration of 1 μ M, 4 fmol mRNA could not be differentiated from background. With 5 μ M Ru(bpy), 4 fmol mRNA could be measured but not 400 amol. Best results were obtained at Ru(bpy) concentrations of 10 and 20 μ M where 1 fmol mRNA was clearly visible and 400 amol mRNA was detected on some ITO electrodes.

Influence of flow rate:

Flow rates of 0.25, 0.5 and 1 ml/min were investigated. Flow rate was found to have little influence on the limit of detection of the technique or on the intensity of the signal. Overall, faster flow rates seemed to give slightly higher signals.

Influence of ITO:

Some electrode-to-electrode variations were observed within the same ITO lot and among different ITO lots. Preliminary results suggest these variations apply to both signal intensity and limit of detection.

Short oligo vs. mRNA:

Under identical experimental conditions long target exhibited a lower limit of detection than short oligonucleotide. Using 20 μ M Ru(bpy), the lowest detectable amount of a short 51-mer DNA with 17 Gs was 1 pmol while it was 1 fmol for the longer ApoA1 mRNA.

Sampling Rate:

Sampling rates of 2 and 10 points per second (pt/s) were used. At 10 pt/s the baseline was unstable and 1 fmol ApoA1 mRNA could barely be detected. To date, best results with large electrodes were obtained at a sampling rate of 2 pt/s.

Sample Preparation:

It was found that vortexing the sample immediately prior to injection profoundly influenced the results. Samples vortexed immediately prior to injection gave more signal than samples that were left to rest. Vortexing may also allow better discrimination from background.

Injection Loop Volume:

The volume of the injection loop was varied from 5 to 50 μL . Volume loops of 5 and 10 μL gave scattered results and poor discrimination between 10 fmol mRNA and the Ru(bpy) background. A 50 μL loop tightened the results but gave poor discrimination between the signal and the background. To date, the loop volume that gave the best results remains 20 μL .

To simplify the detector design, ITO electrodes were derivatized with Ru(bpy) prior to use. Ru(bpy) immobilization utilized the same silane chemistry employed for coupling and immobilization of probe in the Hybridization Module. An $-\text{CH}_2\text{NH}_2$ linker was attached to Rubpy and then coupled to the isocyanato silane using the standard coupling conditions. Silane coupled Ru(bpy) was applied to the ITO electrodes, incubated and electrodes washed following the standard protocol. Data representative of results obtained using immobilized Ru(bpy) are shown in Figure 5. When Ru(bpy) is immobilized, background signal is reduced eliminating the signal from detection buffer alone. This reduction in background simplifies observation of the specific signal generated by the catalytic oxidation of guanine present in the nucleic acid target.

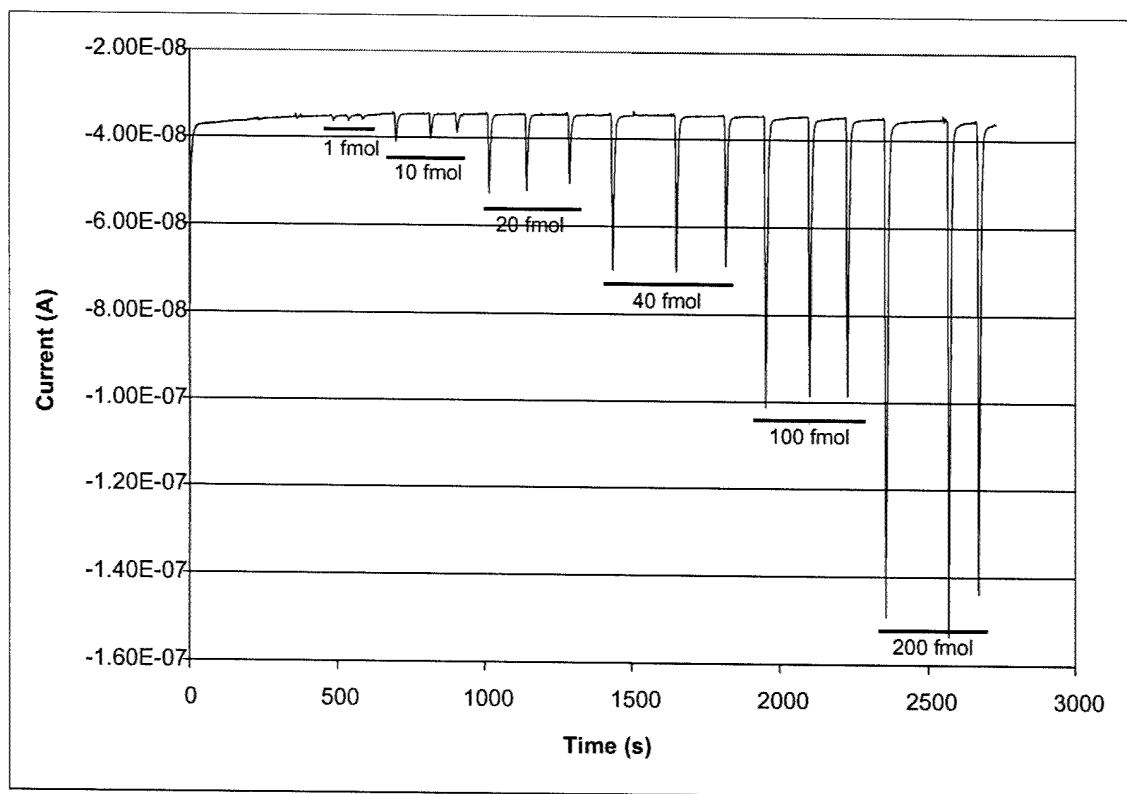


Figure 5. Detection of the ApoA1 target in the flow-through system with Ru(bpy) immobilized at the electrode surface.

Ru(bpy)-silane deposition parameters were examined to determine their effect on the signal observed and the reproducibility of the measurement. Stability of immobilized Rubpy deposited under optimal conditions was examined and it was determined that electrodes used for up to three days still show satisfactory performance.

In conclusion, research on this project has shown great promise for development of a portable DNA biosensor unit. Both the Hybridization and Detection Modules performed well in the proof-of-principle studies and the processes used in the module fabrication and development are simple and easy to implement. It can be expected that joining these modules on a microfluidic platform will result in a powerful DNA or RNA detection unit.

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